APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2006, p. 28–36 Vol. 72, No. 1
0099-2240/06/$08.00+0 doi:10.1128/AEM.72.1.28–36.2006

Role of Autochthonous Filamentous Fungi in Bioremediation of a Soil Historically Contaminated with Aromatic Hydrocarbons

A. D'Annibale,[1] F. Rosetto,[2] V. Leonardi,[1,2] F. Federici,[1] and M. Petruccioli[1]*

Dipartimento di Agrobiologia e Agrochimica, University of Tuscia, Viterbo, Italy,[1] and Interuniversitary Consortium "Chemistry for the Environment," Venezia, Italy[2]

Received 27 May 2005/Accepted 19 September 2005

Nine fungal strains isolated from an aged and heavily contaminated soil were identified and screened to assess their degradative potential. Among them, *Allescheriella* sp. strain DABAC 1, *Stachybotrys* sp. strain DABAC 3, and *Phlebia* sp. strain DABAC 9 were selected for remediation trials on the basis of Poly R-478 decolorization associated with lignin-modifying enzyme (LME) production. These autochthonous fungi were tested for the abilities to grow under nonsterile conditions and to degrade various aromatic hydrocarbons in the same contaminated soil. After 30 days, fungal colonization was clearly visible and was confirmed by ergosterol determination. In spite of subalkaline pH conditions and the presence of heavy metals, the autochthonous fungi produced laccase and Mn and lignin peroxidases. No LME activities were detected in control microcosms. All of the isolates led to a marked removal of naphthalene, dichloroaniline isomers, *o*-hydroxybiphenyl, and 1,1′-binaphthalene. *Stachybotrys* sp. strain DABAC 3 was the most effective isolate due to its ability to partially deplete the predominant contaminants 9,10-anthracenedione and 7H-benz[DE]anthracen-7-one. A release of chloride ions was observed in soil treated with either *Allescheriella* sp. strain DABAC 1 or *Stachybotrys* sp. strain DABAC 3, suggesting the occurrence of oxidative dehalogenation. The autochthonous fungi led to a significant decrease in soil toxicity, as assessed by both the *Lepidium sativum* L. germination test and the Collembola mortality test.

Biological treatment technologies for the remediation of soils and groundwater contaminated with organopollutants are widely used for their environmentally friendly impact combined with low cost compared to other treatment alternatives (42, 47).

An alternative to the biostimulation of indigenous microflora, the so-called practice of bioaugmentation, can favor contaminant degradation when dealing with historically and/or heavily contaminated sites (49). Sites contaminated by recalcitrant organic compounds have often been shown to be characterized by the concomitant presence of heavy metals (11). In such a difficult case, the use of filamentous fungi (white-rot fungi, in particular) may give some advantages over bacterial bioaugmentation (23, 30, 41). Fungi display a high ability to immobilize toxic metals by either insoluble metal oxalate formation, biosorption, or chelation onto melanin-like polymers (8). Moreover, due to the low substrate specificity of their degradative enzyme machinery (e.g., laccase, lignin peroxidase, and Mn peroxidase), fungi are able to perform the breakdown of a wide range of organopollutants in contaminated soils (20, 23). The original belief that ligninolytic peroxidase production is a unique prerogative of white-rot basidiomycetes is being denied by an increasing number of studies reporting the presence of these enzymes in other fungal taxonomic groups (7, 39, 40).

The majority of mycoremediation studies have been performed on artificially contaminated soils spiked with organic pollutants (37, 41). For this reason, it is of paramount importance to investigate the use of fungal remediation under nonsterile conditions and with soils from real contaminated sites, thus making the studies potentially transferable to a field scale (41).

In a previous work (16), the technical feasibility of ex situ bioaugmentation with allochthonous fungi of an aged contaminated soil from the ACNA (Azienda Colorifici Nazionali Affini) site was assessed. This site is a former industrial area where large-scale production of a wide array of organic chemicals had taken place for more than 100 years, until the site was decommissioned in 1994. This historically contaminated soil was characterized by the concomitant presence of aromatic hydrocarbons, including chlorinated benzenes and anilines, thiophenes, and polyaromatic hydrocarbons, and heavy metals.

Within the framework of a coordinated project (Sisifo Project), various approaches to the remediation of the ACNA site were experimented with and compared. Among them, biostimulation of indigenous prokaryotes turned out to be unsuccessful, despite the presence of bacteria specialized in the catabolism of several aromatic compounds (10).

This failure prompted us to assess the eventual presence of yeasts and fungal microbiota adapted to the historical contamination of that site with the objective of using them for its remediation. The bioaugmentation variant consisting of the isolation of indigenous fungi from a contaminated site followed by their reinoculation therein has been reported to be technically feasible and promising (6, 38). The aim of the present work was to screen for the aromatic hydrocarbon-degrading potential of fungal strains isolated from the ACNA soil and to assess the possible use of such selected autochthonous fungi in an ex situ soil biotreatment via bioaugmentation.

* Corresponding author. Mailing address: Dipartimento di Agrobiologia e Agrochimica, University of Tuscia, Via S. C. De Lellis, 01100 Viterbo, Italy. Phone: 39 0761 357332. Fax: 39 0761 357242. E-mail: petrucci@unitus.it.

MATERIALS AND METHODS

Materials. The aged contaminated soil used in this study was from a large decommissioned chemical industrial site (ACNA, Savona, Italy). The air-dried and sieved (<2 mm) soil had pHs of 7.43 and 7.35 in water and 1 N KCl

TABLE 1. Radial growth and dye-decolorization abilities (halo diameters) of fungal isolates grown on PDA supplemented with Poly R-478 (0.01% [wt/vol]) and incubated at different temperatures[a]

Isolate	Mycelial growth[b] at:			Decolorization halo (diameter [mm]) at:		
	24°C	28°C	32°C	24°C	28°C	32°C
Allescheriella sp. strain DABAC 1	++	++	+	0	12.0 ± 0.0	0
Stachybotrys sp. strain DABAC 2	++	++	++	0	11.0 ± 0.1	0
Stachybotrys sp. strain DABAC 3	++	++	++	42.0 ± 1.0	41.5 ± 0.5	42.0 ± 0.0
Allescheriella sp. strain DABAC 4	++	++	+	0	38.5 ± 0.5	0
Allescheriella sp. strain DABAC 5	++	++	+	0	0	0
Allescheriella sp. strain DABAC 6	++	++	+	0	0	0
Allescheriella sp. strain DABAC 7	++	++	+	0	0	0
Aspergillus sp. strain DABAC 8	++	+	+	0	0	0
Phlebia sp. strain DABAC 9	++	+++	+++	55.0 ± 1.0	90[c]	90[c]

[a] Data are means ± standard deviations of five replicates.
[b] The symbols +, ++, and +++ indicate diameters of <30, 30 to 60, and >60 mm, respectively.
[c] Mycelial growth and the decolorization halo covered the whole plate size.

(potential acidity), respectively. The total organic carbon, total nitrogen, and total phosphorous contents were 38.6, 1.2, and 4.8 g kg^{-1} soil, respectively. Soil texture was as follows: sand, 19.36%; silt, 77.14%; and clay, 3.50% (wt/wt) (16). The soil was heavily contaminated by substituted mono- and polycyclic aromatic hydrocarbons (PAHs) and various heavy metals (16).

An air-dried and sieved (<2 mm) noncontaminated hill soil was used as a diluent for ecotoxicological analyses. It had pHs of 6.4 and 5.1 in water and 1 N KCl, respectively, and its contents of sand, silt, and clay were 64.63, 22.90, and 12.47% (wt/wt), respectively. The total organic carbon content was about 15.0 g kg^{-1} soil, while the water holding capacity was 25.49%.

Isolation and identification of autochthonous fungi. Autochthonous yeasts and filamentous fungi were isolated from a suspension obtained by adding 9 ml sterile physiological solution to 1 g soil and magnetically stirring the solution for 30 min. For the isolation of both yeasts and filamentous fungi, aliquots of the suspension were serially diluted and spread onto petri dishes containing the following reagents (g $liter^{-1}$): Rose Bengal agar (Oxoid, Basingstoke, United Kingdom), 16.0; chloramphenicol, 0.1; and streptomycin sulfate, 0.55. To isolate white-rot fungi, the previously described solid medium was supplemented with 0.015 g $liter^{-1}$ benomyl (18). Agar plates were incubated at 28°C for time periods ranging from 5 to 12 days. Isolates were identified according to general principles of fungal classification (19). Due to the difficulty in identifying one of the isolates (white-rot basidiomycete DABAC 9), a molecular approach was used. The DNA was extracted with a Nucleospin plant kit (M-Medical Genenco, Florence, Italy), and the rRNA genes were amplified by PCR using the universal fungal primers NS1, NS3, NS4, NS5, NS8, ITS1, ITS4 (51), ITS4A, and ITS5 (34). The optimal conditions for PCR amplification were as follows: initial denaturation for 2 min at 95°C, with subsequent denaturation for 30 s at 95°C, primer annealing for 30 s at 55°C, and extension for 30 s at 72°C for a total of 35 cycles. A final extension step at 72°C for 5 min ended amplification. The purified PCR products were used in sequencing reactions with the same set of primers, using a BigDye Terminator cycle sequencing ready reaction kit, version 3.0 (Applied Biosystems, Foster City, Calif.). Sequencing was performed on an ABI 3100 sequencer (Applied Biosystems). Sequence (18S rRNA) alignments were carried out by means of the ClustalW 1.83 program, while phylogenetic analysis was performed using the neighbor-joining method in the Phylip 3.6a3 package, both of which are freely available online (http://bioweb.pasteur.fr/). Compared sequences were selected from an initial BLAST search of the database. Bootstrap and parsimony tests guaranteed the reliability of phylogenetic analysis.

The isolates used for this study, listed in Table 1, were maintained at 4°C on potato dextrose agar (PDA; Difco, Detroit, Mich.) and subcultured every month.

Inoculum preparation. Mycelium fragments from 10-day-old PDA culture slants were suspended in sterile deionized water (5 ml) using a sterile Potter homogenizer. Erlenmeyer flasks (500 ml) containing 95 ml of potato dextrose broth (PDB; Difco) were inoculated with these cell suspensions (5 ml per flask) and incubated on a rotary shaker (180 rpm, 28°C) for 96 h. The precultures were centrifuged (4,000 × *g*, 10 min) and washed with deionized water. Deionized water was added to the washed mycelium, and the mixture was homogenized with an Ultra-Turrax macerator (IKA Labortechnik, Germany) (two subsequent steps of 20 s each, at ca. 7,000 rpm) to yield a biomass concentration of approximately 10 g $liter^{-1}$, which was then used to inoculate liquid media or soil.

Screening of isolates on solid and liquid media. Estimations of fungal dye-decolorization abilities were performed with both plate and liquid cultures, using PDA and PDB, respectively, to which Poly R-478 (0.01% [wt/vol]) was added. In the former case, decolorization was determined by measuring the diameter of the yellow halo around the fungal colony. For liquid cultures, decolorization was determined spectrophotometrically by measuring the A_{520}/A_{350} ratio.

To assess the eventual production of lignin-modifying enzymes (LMEs), further experiments were conducted in 500-ml Erlenmeyer flasks containing 95 ml PDB supplemented with veratryl alcohol (VA), guaiacol (G), or maize straw (MS). VA and G were added at 0.25, 2.5, or 25.0 mM, while MS was added at 0.5 or 2.0 g $liter^{-1}$. Flasks were inoculated (5% [vol/vol]), and incubations were carried out at 28°C on a rotary shaker (180 rpm) for 15 days. Culture samples were withdrawn on a daily basis and centrifuged (4,000 × *g*, 10 min), and the supernatants were used for decolorization and enzyme assays (see below). Experiments were carried out in triplicate.

Enzyme extraction and assays. Soil microcosms and soil colonized by fungi underwent extraction at 5°C for 1 h, using a modified procedure described elsewhere (14). Three grams of soil was extracted with 30 ml 0.1 M acetate buffer, pH 5.0 (buffer A), containing 0.005 M $CaCl_2$, 0.05% Tween 80, and 3% insoluble polyvinylpolypyrrolidone. The aqueous suspension was centrifuged (11,000 × *g*, 30 min), and the resulting supernatant was extensively dialyzed with buffer A and concentrated 20-fold in a stirred cell equipped with a PM 30 Diaflo membrane (Amicon). Extracellular soluble protein was determined by the Bradford method (12).

Laccase- and Mn-dependent peroxidase (MnP) activities were determined as previously described (22). Aryl alcohol oxidase and lignin peroxidase (LiP) activities were determined as previously reported (16). All activities were expressed in international units (IU), with 1 IU defined as the amount of enzyme which produces 1 μmol of product per minute under the assay conditions used. Appropriate controls were carried out with heat-denatured extracts.

Soil biotreatment. The contaminated soil was amended with milled (1-mm average length) maize stalks (20% [wt/wt]) and placed in 500-ml Erlenmeyer flasks (40 g of amended soil each). The initial moisture content of the stalk-soil mixture was 7.73%. Twenty milliliters of the above-mentioned fungal suspensions was used to inoculate the mixtures to reach a final soil moisture content of about 36.8%. The flasks were incubated for 30 days with continuous aeration (3 to 5 ml min^{-1} g^{-1} soil) with moistened and nonsterile air at 28°C in the dark. Noninoculated controls (with or without added stalks) were incubated as described above and from here on are referred to as amended and nonamended control microcosms, respectively. All the experiments were carried out in duplicate and under nonsterile conditions.

Determination of mycelial growth and viable heterotrophic bacteria. Fungal growth in plate cultures was determined by measuring radial growth. As for liquid shaken cultures, mycelial pellets were recovered by filtration on a nylon net (2-mm pores) and extensively washed with deionized water prior to determination of their dry weight at 105°C. In addition, ergosterol was used as a specific indicator of fungal growth in soil biotreatment experiments. Extraction and high-performance liquid chromatography determination of ergosterol were carried out as reported by Novotný et al. (35).

Bacterial counts at the start and end of incubation were performed by a modified procedure described by Fava and Bertin (21) with both control microcosms and fungus-treated soils. The sole modification was that cycloheximide (0.5 mg $liter^{-1}$) was added to plate count agar (Oxoid) in order to inhibit fungal growth.

Extraction and analysis of contaminants. Amended control microcosms and soil which had been incubated with fungi underwent Sohxlet extraction with hexane-acetone (3:1 [vol/vol]) for 12 h (35). Two internal standards (2,4,6-trichloroaniline and anthracene-d10) were added to the samples prior to extraction. The extracts were dehydrated with Na_2SO_4 and dried under vacuum at 25°C. The solid residues were weighed, dissolved in ethyl acetate to yield a concentration of 4 mg ml^{-1}, and then analyzed by gas chromatography-mass spectrometry (GC-MS). Mass spectra were recorded by the use of a QP-5050 (Shimadzu, Japan) spectrometer equipped with an AT 20 capillary column (0.25-mm internal diameter by 25 m) (Alltech) at 100 to 280°C with an isothermal program of 100°C for 5 min, 10°C min^{-1} up to 280°C, and finally an isothermal step at 280°C for 30 min. The carrier gas was helium at a linear flow rate of 27 cm s^{-1}. The temperature of both the injection port and the mass interface was 280°C. The identification of contaminants was based on comparison with mass spectra and retention times of pure standards.

For chloride ion determination, each soil sample was added to double-distilled water to obtain a slurry (25% [wt/wt]) and agitated in an orbital shaker (200 rpm for 2 days). The suspension was centrifuged (11,000 × *g*, 15 min), and the supernatant was filtered with a Minisart 0.45-μm syringe filter (Sartorius, Göttingen, Germany). The resulting filtrate was analyzed by the method of Florrence and Farrar (24).

Ecotoxicological analyses. Control microcosms and fungus-treated soil samples were analyzed for their toxicity by using two test organisms, i.e., watercress (*Lepidium sativum* L.) and the Collembola member *Folsomia candida* (Willem). Each soil sample was analyzed both as a whole and at various dilutions with the previously described hill soil.

Static germinability assays with watercress seeds were conducted in a germination chamber for 24 h at 25 ± 1°C in the dark. A randomized complete block experimental design with six replicates and 25 seeds per petri dish was used. For each experiment, 5 g of either control or contaminated soil and 8 ml distilled water were placed into a polystyrene petri dish (60 × 10 mm). A Whatman no. 1 ashless filter paper disk was placed over each sample and then seeded. Germinability tests conducted in the presence of distilled water (controls) were also run in parallel. At the end of the incubation, the number of seeds germinated was counted, and the root lengths were measured by means of a ruler to the closest millimeter. Two different end points were quantified in order to evaluate the effect of tested samples on plant physiological processes, namely, seed germination and root elongation. In order to provide an integrative interpretation, seed germination and root elongation were combined into an index of germination (Ig) according to the following equation: $Ig = (G_sL_s)/(G_cL_c)$, where G_s and L_s are the seed germination and root elongation (mm) values, respectively, for the sample, and G_c and L_c are the corresponding values for the distilled water controls.

The acute toxicity test with *Folsomia candida* was performed as follows. Twenty grams of soil was put into a 50-ml glass container, humidified by the addition of ca. 5 ml of distilled water, and inoculated with 10-day-old subadult springtail Collembola insects. The flasks were closed with an air-permeative plastic cover and incubated in a dark chamber at room temperature for 14 days. The toxic effect was determined by calculating the percent mortality of the adults for each soil concentration and for control microcosms, as reported by Fava and Bertin (21).

Statistical analysis. One-way analysis of variance was applied to percentage data for mortality and germinability, previously transformed into arc sine values of the square root. The significance of the difference between homogeneous data was assessed according to Tukey's multiple comparison test.

Nucleotide sequence accession numbers. The nucleotide sequences obtained in this study have been deposited in GenBank (http://www.ncbi.nlm.nih.gov). The accession numbers for the 18S rRNA gene sequence and the IT1, 5.8S, IT2, and 28S (partial) sequences are AY943950 and AY947835, respectively.

RESULTS

Autochthonous fungus isolation and identification. Plating of serial soil dilutions on selective agar medium followed by incubation and colony counting showed the absence of yeast and/or yeast-like organisms, while it resulted in the isolation of several isolates of filamentous fungi. Growth on a specialized agar medium containing the benzimidazole derivative benomyl allowed the selective growth of putative white-rot basidiomycetes.

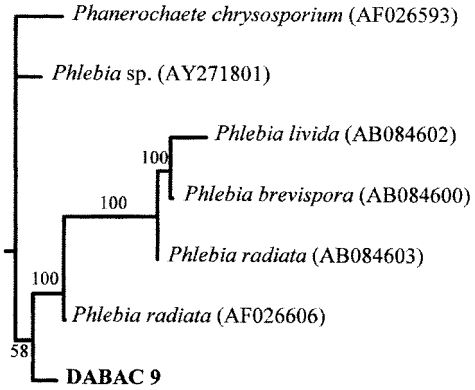

FIG. 1. Neighbor-joining distance-based phylogenetic tree of 18S rRNA gene sequences. *Phanerochaete chrysosporium* was used as the outgroup. Bootstrap values higher than 50% from 1,000 replications are shown on appropriate branches. The basidiomycete isolate DABAC 9 sequence (AY943950) is shown in bold, while accession numbers of compared sequences are reported in parentheses.

Taxonomic examination of isolated fungi on the basis of morphological features and/or identifiable asexual spores (conidia) led to the identification of one *Aspergillus* sp. isolate (DABAC 8) and two and five isolates belonging to the genera *Stachybotrys* (DABAC 2 and 3) and *Allescheriell*a (DABAC 1, 4, 5, 6, and 7), respectively. Resistance to benomyl, the presence of clamp connections at septa, and positive staining with diazonium blue B provided definite evidence of the basidiomycete identity of isolate DABAC 9. However, in the absence of sexual fruiting bodies, its taxonomic identification was done by comparison of its 18S rRNA sequence with sequences of known species. The obtained 18S sequence had the expected length (1,793 bp) and matched at a high rate (similarity of >98%) those of rRNA genes of various species belonging to the genus *Phlebia*, such as *P. radiata* (AB 084603 and AF026606), *P. brevispora* (AB084600), and *Phlebia* sp. (AY271801). Figure 1 shows a phylogeny tree built on the sequence alignments, using *Phanerochaete chrysosporium* (AF026593) as the outgroup. On this basis, DABAC 9 might be classified as a *Phlebia* sp. strain.

Screening of autochthonous fungi. All of the fungal isolates were preliminarily screened for their hydrocarbon-degrading potential on PDA plates containing the anthraquinone-based dye Poly R-478. Table 1 reports the radial growth and diameters of the decolorization halo obtained with each of the isolates incubated at three different temperatures. Regardless of the incubation conditions, three *Allescheriella* sp. (DABAC 5, 6, and 7) and one *Aspergillus* sp. (DABAC 8) isolate proved to be unable to perform dye decolorization. The most effective and versatile isolates were *Phlebia* sp. strain DABAC 9 and *Stachybotrys* sp. strain DABAC 3, with the former one leading to complete dye decolorization at 28 and 32°C (Table 1).

The same isolates were further screened for decolorization ability and LME production in shaking cultures at 28°C. Dye decolorization was calculated by the reduction in the A_{520}/A_{350} ratio rather than from the wavelength of maximal absorbance of the dye (i.e., 520 nm) to avoid artifacts due to Poly R-478 adsorption to the mycelial cell walls. Regardless of the isolate, laccase activity was the sole LME activity detected under the

TABLE 2. Mycelial growth, dye-decolorization ability, and laccase activity production by fungal isolates cultivated in shaking cultures in PDB supplemented with Poly R-478 (0.01% [wt/vol])[a]

Strain	Decolorization[b] (A_{520}/A_{350})[c]	Laccase activity[b] (mIU ml^{-1})	Mycelial growth[d] (g liter^{-1})
Allescheriella sp. strain DABAC 1	0.18 ± 0.01 (11)	41.5 ± 3.6 (13)	11.0 ± 0.3
Stachybotrys sp. strain DABAC 2	0.28 ± 0.01 (14)	12.5 ± 1.1 (9)	6.6 ± 1.1
Stachybotrys sp. strain DABAC 3	0.24 ± 0.02 (14)	31.5 ± 2.0 (5)	6.1 ± 0.6
Allescheriella sp. strain DABAC 4	0.17 ± 0.02 (11)	25.5 ± 5.1 (7)	6.4 ± 1.8
Allescheriella sp. strain DABAC 5	0.36 ± 0.02 (11)	ND[e]	8.4 ± 1.2
Allescheriella sp. strain DABAC 6	0.24 ± 0.01 (11)	ND[e]	14.0 ± 1.1
Allescheriella sp. strain DABAC 7	0.25 ± 0.01 (9)	ND[e]	9.4 ± 0.8
Aspergillus sp. strain DABAC 8	0.22 ± 0.00 (9)	ND[e]	10.8 ± 1.0
Phlebia sp. strain DABAC 9	0.16 ± 0.01 (13)	109.3 ± 2.8 (7)	5.9 ± 0.8

[a] Data are means ± standard deviations of three independent experiments.
[b] Reported data are for maximal values of decolorization or laccase activity, and numbers in parentheses indicate the time (days) required to attain those values.
[c] The initial A_{520}/A_{350} ratio was 0.460 ± 0.04.
[d] Mycelial growth is given as g of dry weight per liter of culture broth after 15 days of fermentation.
[e] ND, no detectable activity.

tested conditions (Table 2). The best results were obtained with *Phlebia* sp. strain DABAC 9, which led to an A_{520}/A_{350} ratio of 0.16 ± 0.01 (about 65% dye decolorization) and to the highest laccase activity (109 ± 2.8 mIU ml^{-1}). However, two *Allescheriella* sp. (DABAC 1 and 4) and two *Stachybotrys* sp. (DABAC 2 and 3) isolates gave promising results, albeit at a lesser extent than *Phlebia* sp. strain DABAC 9.

These five isolates were tested for their response in shaken cultures to two lignin-related compounds (i.e., VA and G) and milled maize stalks (MS), with the latter being used as an amendment in the subsequent mycoremediation experiments. Figure 2A and B show that both VA and G, when added at 25 mM, led to complete growth suppression of *Allescheriella* sp. isolates. In contrast, *Stachybotrys* sp. strain DABAC 3 and *Phlebia* sp. strain DABAC 9 proved to be highly tolerant to such a high VA concentration, even though mycelial growth was reduced by 19 and 30%, respectively, with respect to that in PDB alone (Fig. 2A). The addition of 0.2% finely milled maize stalks resulted in markedly more fungal growth of *Stachybotrys* DABAC 3 (8.7 versus 4.3 g liter^{-1}) and *Phlebia* sp. strain DABAC 9 (9.5 versus 2.5 g liter^{-1}) than that obtained on PDB alone (Fig. 2C).

Regardless of the supplement (VA, G, or MS) and the fungal isolate, laccase activity was the sole LME activity detected under the tested conditions. Laccase activity was not produced by *Allescheriella* sp. isolates in the absence of the supplements but was detected when 0.25 mM VA was added to the growth medium (32.5 ± 1.4 and 133.3 ± 11.4 mIU ml^{-1} for DABAC 1 and DABAC 4, respectively) (Fig. 3A). In contrast, laccase activity was detected in *Stachybotrys* sp. strain DABAC 2 and DABAC 3 and *Phlebia* sp. strain DABAC 9 cultures even in the absence of the aforementioned supplements (11.7 ± 1.2, 21.2 ± 1.8, and 121.0 ± 6.6 mIU ml^{-1}, respectively). Laccase production by *Phlebia* sp. strain DABAC 9 increased with the VA concentration, reaching a maximum (1,358.0 ± 98.7 mIU ml^{-1}) at 25 mM. It is worth pointing out that the use of mineral elements (i.e., copper and manganese ions) and/or alternative liquid media such as malt extract broth did not lead to the onset of other LME activities such as MnP and LiP activities (data not shown). In addition, the use of Kirk's medium (45) under conditions of low and high nitrogen content (2 and 20 mM, respectively) resulted in poor growth of the tested strains and, consequently, a lack of LME activities (data not shown).

Contaminated soil colonization. Soil was completely colonized within the first 3 weeks of incubation by the *Stachybotrys* sp. strain DABAC 3 isolate, as assessed by visual inspection. In contrast, *Phlebia* sp. strain DABAC 9 and *Allescheriella* sp. strain DABAC 1 exhibited slower growth. This was confirmed by the quantification of ergosterol, a sterol specifically used to quantify fungal biomass (35). The ergosterol content in soil treated for 30 days with either *Allescheriella* sp., *Stachybotrys* sp., or *Phlebia* sp. was 5.2 ± 0.6, 30.5 ± 3.6, or 10.7 ± 2.4 μg g^{-1} soil, respectively. No ergosterol was detected in the amended control microcosm.

Despite the marked differences in the extent of mycelial growth, soluble protein production levels observed in acetate-buffered extracts of soil colonized by *Allescheriella* sp., *Stachybotrys* sp., and *Phlebia* sp. did not differ significantly from one another (423.7 ± 21.4, 507.2 ± 41.0, and 434.5 ± 19.1 μg g^{-1} soil, respectively). The soluble protein content of the amended control microcosm was 12.8 ± 0.9 μg g^{-1} soil.

The concentrations of indigenous cultivable aerobic bacteria in the nonamended contaminated soil before and after 30 days of incubation (control microcosm) did not differ significantly (Fig. 4). Soil amendment with milled maize stalks significantly stimulated bacterial growth (Fig. 4). In soil which had been incubated with either *Allescheriella* sp., *Stachybotrys* sp., or *Phlebia* sp., the concentrations of heterotrophic viable bacteria were significantly higher than that in the amended control microcosm (Fig. 4).

Bioaugmentation of soil with either *Allescheriella* sp., *Stachybotrys* sp., or *Phlebia* sp. led to a slight pH reduction (final pH values, 7.28, 7.20, and 7.42, respectively). In contrast, the pH was shifted towards more alkaline values (7.82) in the amended control microcosm.

LME activities in contaminated soil. Several lignin-modifying oxidase activities were detected in soil colonized by fungi, as shown in Table 3. However, none of the isolates produced aryl alcohol oxidase. The highest LiP activity was detected in the acetate-buffered extract of the soil treated with *Phlebia* sp. strain DABAC 9 and amounted to 83.1 ± 2.2 mIU g^{-1} soil. MnP activity was produced by all of the isolates, with the

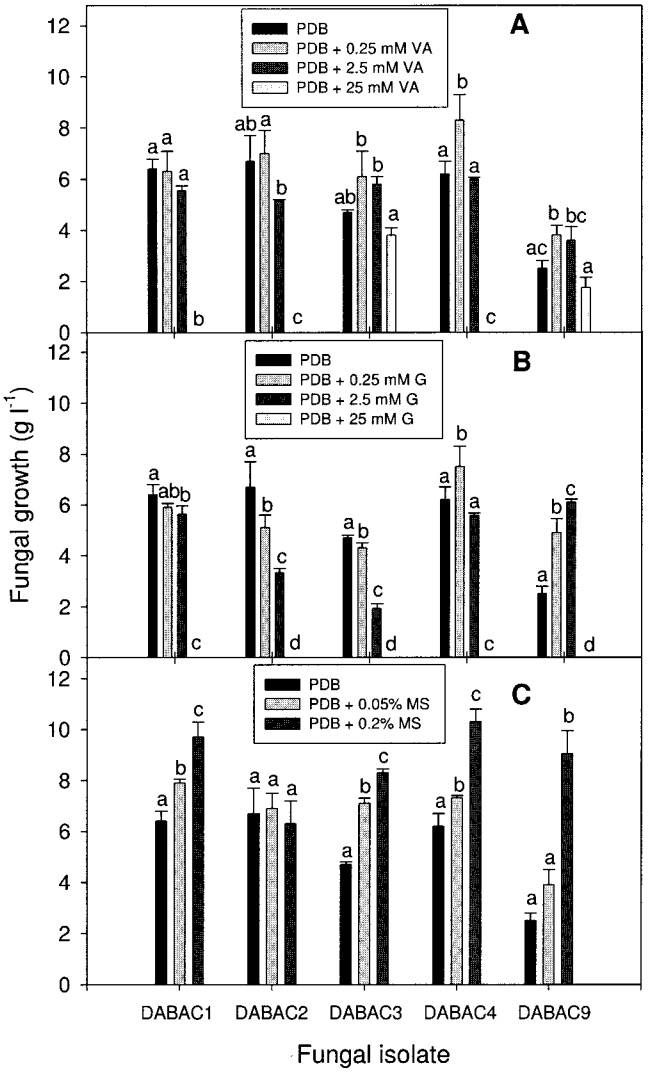

FIG. 2. Fungal growth of *Allescheriella* sp. (isolates DABAC 1 and DABAC 2), *Stachybotrys* sp. (isolates DABAC 3 and DABAC 4), and *Phlebia* sp. (DABAC 9) cultured on PDB in the presence of either VA (A), G (B), or MS (C). VA and G were added at 0.25, 2.5, and 25 mM, while MS was added at 0.05 and 0.2% (wt/vol). Cultures were performed at 28°C with orbital shaking (180 rpm), and biomass was determined on day 15. Values are the means of three independent experiments, and error bars indicate standard deviations. Statistical pairwise multiple comparisons of data were carried out by the Tukey test: for the same fungal isolate, mean values with the same superscript letter were not significantly different ($P \leq 0.05$).

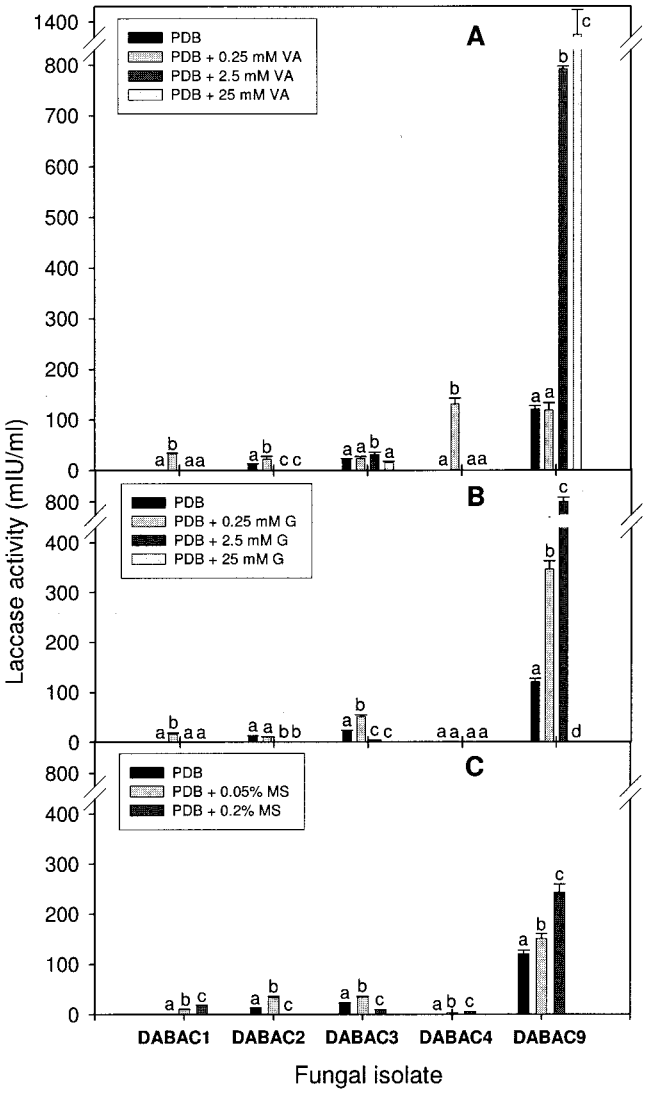

FIG. 3. Laccase activities of *Allescheriella* sp. (isolates DABAC 1 and DABAC 2), *Stachybotrys* sp. (isolates DABAC 3 and DABAC 4), and *Phlebia* sp. (DABAC 9) cultured on PDB in the presence of either VA (A), G (B), or MS (C). VA and G were added at 0.25, 2.5, and 25 mM, while MS was added at 0.05 and 0.2% (wt/vol). Cultures were performed at 28°C for 15 days with orbital shaking (180 rpm). Values are given for the time of maximal enzyme production and represent the means of three independent experiments, and error bars indicate standard deviations. Statistical pairwise multiple comparisons of data were carried out by the Tukey test: for the same fungal isolate, mean values with the same superscript letter were not significantly different ($P \leq 0.05$).

highest level (20 ± 0.9 mIU g^{-1} soil) being produced by *Stachybotrys* sp. strain DABAC 3. Among the LMEs produced in remediation trials, laccase exhibited the lowest activity. LME activities were not detected in the amended control microcosm.

Organopollutant degradation in soil. Table 4 reports the fungal decontamination of soil, expressed as percentages of reduction of pollutant concentrations with respect to those in the amended control microcosm, taken as a reference. As a matter of fact, some organopollutants initially present in the amended contaminated soil (e.g., tetrachlorothiophene, phenanthrene, pentachlorobenzene, and diphenylsulfone) were not detected in the amended control microcosm.

All of the autochthonous fungi employed led to a significant removal of naphthalene, dichloroaniline isomers, *o*-hydroxybiphenyl, and 1,1′-binaphthalene. *Stachybotrys* sp. strain DABAC 3 was the most effective isolate due to its ability to bring about partial depletion of the predominant contaminants 9,10-anthracenedione and 7H-benz[DE]anthracen-7-one, with their percentages of removal being about 49.8 and 18.7%, respectively (Table 4). In addition, the *Stachybotrys* sp. isolate also

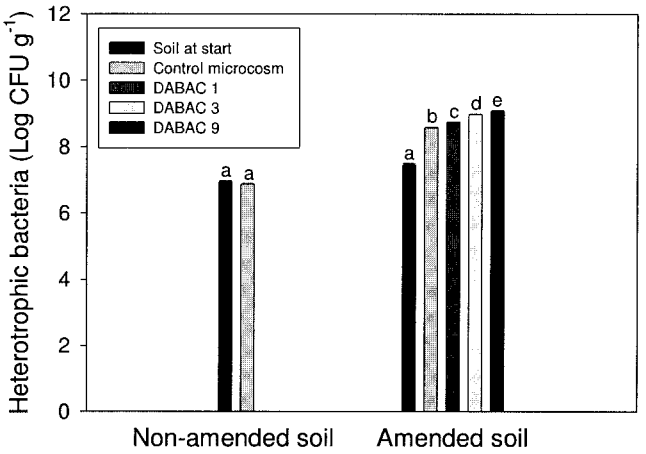

FIG. 4. Concentrations of indigenous cultivable aerobic bacteria in nonamended or amended contaminated ACNA soil at the start of the experiment, in related control microcosms, and in amended soil treated with either *Allescheriella* sp. strain DABAC 1, *Stachybotrys* sp. strain DABAC 3, or *Phlebia* sp. strain DABAC 9. Values represent the means of four determinations (two replicates for two independent experiments), and error bars indicate standard deviations. Statistical pairwise multiple comparisons of data were carried out by the Tukey test: mean values with the same superscript letter were not significantly different ($P \leq 0.05$).

exhibited the unique ability to partially degrade anthracenedione derivatives (i.e., 1-chloro- and 1-amino-9,10-anthracenedione) and 2,3,4,5,6-pentachloroaniline.

Unlike the other two isolates, *Phlebia* sp. strain DABAC 9 was definitely unable to deplete 1,2,4,5-tetrachlorobenzene, diphenyl ether, and 2,6-dichloro-3-methyl-aniline (Table 4).

A significant increase in the amount of chloride ions was observed in the amended control microcosm (138.7 ± 12.1 μg g^{-1} soil) with respect to the amended soil at the start of incubation. A further significant increase in chloride ions was detected in soils colonized by either *Allescheriella* sp. strain DABAC 1 or *Stachybotrys* DABAC 3 (285.9 ± 18.0 and 386.8 ± 27.3 μg g^{-1} soil, respectively) (data not shown). In contrast, the chloride content in soil colonized by *Phlebia* sp. strain DABAC 9 did not significantly differ from that measured in the amended control microcosm (data not shown).

Soil ecotoxicity. The nonamended control microcosm proved to be highly toxic, resulting in 100% mortality for the Collembola *Folsomia candida* (Willem). A fivefold dilution of this sample with a noncontaminated soil led to a toxicity decrease (about 57% mortality), as shown in Fig. 5. With the amended control microcosm, 73% mortality was observed, and 20-fold dilution with a noncontaminated soil resulted in a mortality rate of about 25%.

Soil treatment with the isolates resulted in significant detoxification, with *Stachybotrys* sp. strain DABAC 3 and *Phlebia* sp. strain DABAC 9 being more effective than *Allescheriella* sp. strain DABAC 1 (25.3 ± 3.18 and 26.7 ± 4.18 versus 35.4 ± 2.1% mortality, respectively, when undiluted fungus-treated soil was tested) (Fig. 5).

Lepidium sativum germinability was markedly reduced in both nonamended and amended control microcosms with respect to the distilled water control (48.8 ± 2.4% and 58 ± 1.5%, respectively, versus 85.3 ± 2.7%) (Table 5). In the same samples, radicle elongation was also severely affected (2 mm for both microcosms versus 10.0 mm for the water control). Table 5 shows that all of the isolates were able to significantly increase both the radicle length and the cumulative index of germinability.

DISCUSSION

The majority of bioaugmentation studies of historically contaminated sites have been conducted with allochthonous fungi (37, 41). However, the use of autochthonous species for the cleanup of a contaminated site has been shown to be a successful approach (6, 25, 38, 47, 49). These findings prompted us to investigate this remediation approach.

The capability of performing decolorization of Poly R-478 was the main criterion to preliminarily select isolates from the ACNA soil. Although a low correlation was reported between the extent of decolorization and the degradation of some pesticides (9), decolorization of polymeric dyes such as Poly R-478 has been shown to be a valuable tool for selecting xenobiotic-degrading fungi (23, 36, 43, 52). In this respect, Šašek and collaborators (43), while confirming the adequacy of this screening approach, showed that some species, such as *Phanerocheate chrysosporium*, were able to decolorize Poly R-478 only on ammonium-limited media. For this reason, dye decolorization associated with LME production in the presence of two lignin-related compounds (VA and G) or MS was the ultimate selection criterion employed in the present study. On this basis, three isolates, belonging to the genera *Allescheriella* (DABAC 1), *Stachybotrys* (DABAC 3), and *Phlebia* (DABAC 9), were selected as most promising for ACNA soil remediation. It is worth mentioning that laccase was the sole LME produced in shaken cultures, regardless of the isolate and of the added compound or lignocellulosic waste. From data in the literature, laccase production is a prerogative of the genera to which the selected isolates belong (4, 23, 29).

TABLE 3. Specific activities of lignin-modifying enzymes in the amended control microcosm and in amended soil incubated for the same time (30 days) with either *Allescheriella* sp. strain DABAC 1, *Stachybotrys* sp. strain DABAC 3, or *Phlebia* sp. strain DABAC 9[a]

Expt	Laccase sp act		Mn peroxidase sp act		Lignin peroxidase sp act	
	(mIU g^{-1} soil)	(mIU mg^{-1} protein)	(mIU g^{-1} soil)	(mIU mg^{-1} protein)	(mIU g^{-1} soil)	(mIU mg^{-1} protein)
Control microcosm	ND	ND	ND	ND	ND	ND
Allescheriella sp. strain DABAC 1	5.4 ± 0.6	13.1 ± 1.4	10.3 ± 0.9	30.5 ± 3.0	3.0 ± 0.3	8.8 ± 0.6
Stachybotrys sp. strain DABAC 3	4.5 ± 0.5	9.2 ± 0.3	20.0 ± 0.9	41.8 ± 3.1	3.9 ± 0.4	8.7 ± 0.2
Phlebia sp. strain DABAC 9	3.8 ± 0.3	8.8 ± 0.9	11.4 ± 2.0	27.0 ± 2.2	83.1 ± 2.2	191.5 ± 13.4

[a] Data are means ± standard deviations of four determinations (two replicates for two independent experiments). ND, no detectable activity.

TABLE 4. Concentrations of organic pollutants in amended control microcosm (taken as a reference) and fungal decontamination by either *Allescheriella* sp. strain DABAC 1, *Stachybotrys* sp. strain DABAC 3, or *Phlebia* sp. strain DABAC 9, expressed as percentages of reduction in pollutant concentrations[a]

Contaminant	Concn in control microcosm (mg kg^{-1} soil)	% Reduction by indicated strain		
		DABAC 1	DABAC 3	DABAC 9
Naphthalene	8.99	92.4	91.9	83.3
1,2,4,5-Tetrachlorobenzene	3.57	13.2	0	0
2,6-Dichloroaniline	55.14	80.5	91.5	61.5
2,4-Dichloroaniline	21.46	59.6	75.2	84.3
Diphenyl ether	15.98	37.1	54.0	0
o-Hydroxybiphenyl	18.50	52.1	88.3	88.2
2,6-Dichloro-3-methylaniline	1.60	59.4	65.6	0
2,3,4,5,6-Pentachloroaniline	17.13	0	37.6	0
9,10-Anthracenedione	544.13	5.4	49.8	3.8
1-Chloro-9,10-anthracenedione	3.09	0	17.9	0
1-Amino-9,10-anthracenedione	7.99	0.2	43.5	0
1,1′-Binaphthalene	6.61	58.8	66.2	100.0
7H-benz[DE]anthracen-7-one	1,012.39	2.5	18.7	1.5

[a] Data are means of four chromatographic runs (two replicates for two independent experiments). The standard deviation of the data was <8%.

All of the selected isolates were able to colonize the amended ACNA soil under nonsterile conditions, thus showing good tolerance to high concentrations of toxic contaminants and the ability to compete with the indigenous bacterial microflora. This is not an obvious result, since several fungi displaying a high organopollutant-degrading capability in sterile contaminated soils failed to grow under nonaxenic conditions (30, 32). With this regard, the amendment (i.e., milled maize stalks) employed in the present study turned out to adequately support fungal growth. It should be borne in mind, in fact, that an external nutritional supply is generally required by fungi due to their inability, unlike bacteria, to use organopollutants as a primary C and N source (37, 46). The amendment of nonbioaugmented soil (control microcosm) with maize stalks might be regarded as a biostimulation approach. The observed increase in the viable heterotrophic bacterial concentration in the amended control microcosm confirmed this hypothesis and is in agreement with other studies (13, 33). On the other hand, fungal growth in the amended control microcosm was not significantly stimulated, as inferred by the undetectable ergosterol levels at the final time point (30 days). In this respect, several studies have reported that indigenous fungi, although putatively adapted to their polluted habitat, are frequently present in a metabolically inactive form (13, 41). This might explain why the vast majority of mycoremediation studies rely on bioaugmentation rather than biostimulation.

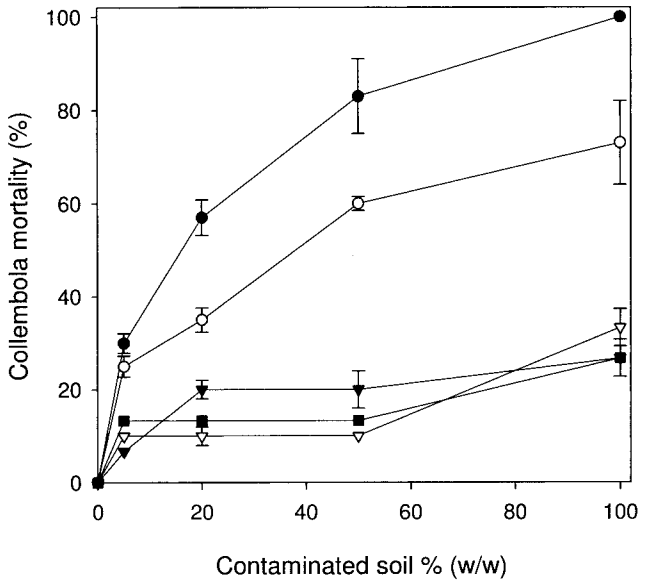

FIG. 5. Collembola mortality percentages as a function of dilution of control microcosms (nonamended [●] or amended with maize stalks [○]) and amended soil incubated for the same length of time (30 days) with either *Allescheriella* sp. strain DABAC 1 (▼), *Stachybotrys* sp. strain DABAC 3 (■), or *Phlebia* sp. strain DABAC 9 (▽). Values represent the means of four determinations (two replicates for two independent experiments), and error bars indicate standard deviations.

Šašek (41) hypothesized that fungi might play the role of launching a primary attack on poorly bioavailable pollutant molecules, thereby detoxifying or converting them into forms that are more susceptible to bacterial degradation. This could account for the observed bacterial growth stimulation in soils colonized by all of the fungal isolates in this study. It is worth noting that several studies have reported that competition among bacteria and fungi generally results in the predominance of the former over the latter (27, 33, 46). However, mycelial colonization by white-rot strains belonging to the genus *Pleurotus* was shown to result in a marked reduction of bacterial growth (27, 33, 37).

Interestingly, both LiP and MnP activities were detected in soil colonized by each of the isolates, although the same isolates were unable to produce ligninolytic peroxidases in shaken cultures. This finding might be due to the smaller amount of ammonium ions in the contaminated soil than in liquid cultures (data not shown), since it is widely known that these heme peroxidases, unlike laccase, are generally produced under nitrogen depletion conditions (26). It is widely known that laccase is able to catalyze the formation of manganic chelates under various conditions (3, 44) and that some phenolic compounds can effectively mediate benzylic alcohol oxidation by this enzyme (15). For this reason, the Azure-B assay (5) and the H_2O_2-independent NADH oxidation method (31) were also employed to confirm the presence of LiP and MnP, re-

TABLE 5. Germinabilities of *Lepidium sativum* L. seeds, radicle lengths, and indexes of germination (Ig) in distilled water control, nonamended and amended control microcosms, and amended soil incubated for the same time (30 days) with either *Allescheriella* sp. (DABAC 1), *Stachybotrys* sp. (DABAC 3), or *Phlebia* sp. (DABAC 9)[a]

Matrix/organism	% Germinability	Radicle length (mm)	Ig
Distilled water/none	85.3 ± 2.7[a]	10.0 ± 0.5[a]	1[a]
Nonamended soil/microcosm	48.8 ± 2.4[b]	2.0 ± 0.2[b]	0.114 ± 0.003[b]
Amended soil/microcosm	58.0 ± 1.5[c]	2.0 ± 0.03[b]	0.136 ± 0.004[c]
Contaminated amended soil/DABAC 1	73.6 ± 2.0[ac]	4.3 ± 0.16[c]	0.371 ± 0.042[c]
Contaminated amended soil/DABAC 3	70.4 ± 5.1[ac]	4.4 ± 0.19[c]	0.363 ± 0.042[c]
Contaminated amended soil/DABAC 9	72.0 ± 5.3[ac]	5.4 ± 0.28[c]	0.455 ± 0.037[c]

[a] Data are the means of six replicates ± standard errors of the means. Statistical pairwise multiple comparisons of homogeneous data were carried out by the Tukey test: column means followed by the same superscript letters were not significantly different ($P \leq 0.05$).

spectively, in the same enzyme extracts (data not shown). To the best of our knowledge, this is the first study reporting the production of both LiP and MnP in the genera *Allescheriella* and *Stachybotrys*. The absence of LiP and MnP activities in shaken cultures of *Allescheriella* sp. strain DABAC 1, *Stachybotrys* sp. strain DABAC 3, and *Phlebia* sp. strain DABAC 9 was likely due to the high stirring speed (180 rpm), considering the negative impact of shear stress on the production of peroxidases (48). In addition, it cannot be ruled out that the nitrogen concentration in the liquid growth medium employed (i.e., PDB) did not allow the expression of ligninolytic peroxidases, taking into account that the onset of these enzyme activities in several fungi occurs under ammonium depletion conditions (20, 23). An attempt to unravel this aspect failed due to the poor growth of the strains of *Allescheriella* sp. and *Stachybotrys* sp. and the subsequent lack of LME production in standard Kirk's medium in the presence of both high and low ammonium contents.

With regard to decontamination of the ACNA soil, all of the isolates employed led to a significant removal of naphthalene, dichloroaniline isomers, *o*-hydroxybiphenyl, and 1,1′-binaphthalene, which are potential candidate substrates for either fungal peroxidases or laccase. In this respect, however, several recent comprehensive reviews reported an increasing number of studies putting in doubt the occurrence of a correlation between LME activities and degradation extents of a number of xenobiotics or pointing to the role of other enzymes, such as cytochrome P-450 monooxygenase and epoxide hydrolase (37, 41). As a matter of fact, several substituted ketone derivatives of PAHs, the degradation of which may not be ascribed to the action of LMEs, were markedly depleted from the ACNA soil.

In particular, *Stachybotrys* sp. strain DABAC 3 was the most effective isolate due to its ability to bring about partial depletion of the predominant contaminants 9,10-anthracenedione and 7H-benz[DE]anthracen-7-one. In addition, anthracenedione derivatives (i.e., 1-chloro- and 1-amino-9,10-anthracenedione) and 2,3,4,5,6-pentachloroaniline were also partially depleted by *Stachybotrys* sp. strain DABAC 3, unlike the case with the other isolates. In this respect, it should be pointed out that several fungal species commonly used for remediation purposes have been found to be unable to degrade ketone derivatives of polyaromatic hydrocarbons (1). In the specific case of 9,10-anthracenedione, its degradation has been shown to often be the rate-limiting step in the mineralization of anthracene (50), and this PAH degradation intermediate was found to accumulate as a dead-end metabolite (2).

A significant release of chloride ions was observed in the present study in soil bioaugmented with *Stachybotrys* sp. strain DABAC 3 and *Allescheriella* sp. strain DABAC 1, likely due to a partial dehalogenation of chlorinated contaminants. This was not surprising since both lignin peroxidase and laccase have been shown to be able to catalyze the oxidative dechlorination of both chlorophenols and chloroanilines (17, 28).

Organopollutant depletion is not a sufficient criterion to evaluate the efficacy of a bioremediation approach, and consequently, a direct determination of the residual toxicity is required (21). In the present study, soil treatment with the isolates resulted in significant detoxification, with *Stachybotrys* sp. strain DABAC 3 and *Phlebia* sp. strain DABAC 9 being more effective than *Allescheriella* sp. strain DABAC 1.

In conclusion, this study confirms that the isolation of fungi from a contaminated soil followed by their reinoculation at the same site can be a valuable remediation strategy.

ACKNOWLEDGMENTS

We thank the interuniversity consortium "Chemistry for the Environment" (I.N.C.A.), which supported, with the contribution of the Italian Ministry of Environment, this work within the Sisifo Project (ACNA—Bonifica di siti contaminati).

We also kindly thank Laura Selbmann (University of Viterbo) for her expert assistance with fungal identification.

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